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Short communication

Non-pet dogs as sentinels and potential synanthropic reservoirs of tick-borne and zoonotic bacteria

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ABSTRACT

Blood samples were collected from 100 shepherd dogs, 12 hunting dogs and 14 stray dogs (apparently healthy) in southern Hungary to screen for the presence of emerging tick-borne pathogens. Based on real-time PCR results, 14 dogs (11%) had single or dual haemoplasma infection, and a same number of samples were positive for *Anaplasma phagocytophilum*. In one sample *Coxiella burnetii* was molecularly identified, and 20.3% of dogs seroconverted to the Q fever agent. Rickettsaemia (sensu stricto) was also detected in one animal. This is the first molecular evidence of autochthonous infection of dogs with the above pathogens in Hungary. The relatively high prevalence of haemoplasma and anaplasma infection among non-pet dogs is suggestive of a prolonged carrier status and bacteraemia of these animals rendering them epidemiologically significant as potential reservoirs and sentinels for tick-borne infections.

1. Introduction

The number of reported cases of tick-borne diseases and the variety of tick-borne agents have been increasing worldwide in recent years (Paddock and Telford, 2010) in part because they represent emerging problems but also because the recently developed high-sensitivity molecular tools allow for more effective detection (Telford and Goethert, 2004). The significance of tick bites and tick-borne infections is a rapidly increasing concern in both veterinary medicine (Fritz, 2009) and human health (Parola and Raoult, 2001).

Living in close association, humans and dogs play a particularly intertwined role in the epidemiology of pathogens transmitted by ticks. In addition to being susceptible to tick-borne agents, dogs may serve as reservoirs of tick-borne human pathogens, as a source of infection for vector ticks, as mechanical transporters of ticks, and as sentinel indicators of regional infection risk (Fritz, 2009). Conversely, pet dogs in developed countries are not only well cared for but are also usually treated against tick bites using preventative measures. Apart from this care, they are usually only sporadically taken on walks and may have less access to alternative infectious sources

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of tick-borne pathogens. Consequently, although the majority of studies on tick-borne and zoonotic canine infections focus on pet dogs (e.g., Kohn et al., 2011), data obtained from this type of sample source may not reflect the real epidemiological situation or actual veterinary-medical health hazards associated with dog-keeping in an endemic region.

To compensate for this inconsistency in the literature, it was decided to molecularly investigate tick-borne and zoonotic pathogens in blood samples from dogs that are either kept extensively (shepherd dogs) or are exposed to tick bites and other infectious sources more often than pet dogs (i.e., hunting and stray dogs).

2. Materials and methods

EDTA-anticoagulated and non-treated blood samples were collected by cephalic venipuncture from 100 shepherd dogs, 12 hunting dogs and 14 stray dogs from 24 locations in south Hungary, during the mid-summer of 2012. All dogs were selected randomly and appeared to be healthy but none were clinically evaluated. Animal data (sex, age; the latter for stray dogs estimated from their dentition) were recorded. EDTA blood samples were frozen at −20 °C until further processing. Sera were separated from non-anticoagulated blood samples after an overnight storage at 4 °C.

DNA was obtained using the QIAamp Mini Kit (Qiagen Inc., Hilden, Germany) individually from 200 μl of blood per sample (adding extraction controls) following the manufacturer's instruction. The quality and quantity of extracted DNA was examined with a TaqMan real-time PCR, which amplifies the canine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene as described previously (Boretti et al., 2009). Samples were screened for haemoplasma infection with a universal SYBR Green real-time PCR adapted from Willi et al. (2009) using an ABI 7500 Fast Sequence Detection System (Life Technologies, Zug, Switzerland) and the KAPA SYBR® FAST qPCR Kit (KAPABiosystems, Boston, USA) with 200 nM of primers. This was followed by species-specific TaqMan real-time PCRs, which detect part of the 16S rRNA gene (Wengi et al., 2008) with dilutions of plasmid DNA of known copy number for quantification purposes.

For comparison with haemoplasma prevalence, *Ehrlichia canis* was also evaluated in the blood samples using a TaqMan real-time PCR that amplifies a portion of the 16S rRNA gene of *E. canis* as described previously (Foley et al., 2007). The presence of *A. phagocytophilum* was investigated with a TaqMan real-time PCR, which detects part of the major surface protein (*msp*) -2 gene as reported (Courtney et al., 2004), but with a modified probe (FAM instead of HEX). The target for *C. burnetii* was the IS1111a gene in a TaqMan real-time PCR (Loftis et al., 2006). Evaluation of *Rickettsia* spp. was conducted using two real-time TaqMan PCRs based on the detection of the 23S gene of *R. helvetica* and detection of the citrate synthase (gltA) gene for other rickettsiae (Boretti et al., 2009).

To detect antibodies to *C. burnetii*, serum samples were diluted at 1:400 and examined by the commercial CHEKIT Q-Fever Antibody ELISA Test Kit (IDEXX, Liebefeld-Bern, Switzerland) using inactivated *C. burnetii* phase 1 and phase 2 antigens. The ELISA was carried out according to the manufacturer's instructions with the anti-ruminant immunoglobulin conjugate replaced with an anti-dog IgG (H + L) HRP-conjugate (1:40,000; Jackson ImmunoResearch Laboratories, Inc.,West Grove, PA, USA). Control samples were chosen and validated in advance with the complement fixation test (current gold standard) as previously reported (Gyuranecz et al., 2012). The ELISA was optimised by measuring linearity, intra-run precision, inter-run precision, analytical sensitivity, recovery, dilution verification and reference range. The optical density (OD) of samples was measured using a plate reader (Labsystems Oy, Helsinki, Finland) at a wavelength of 450 nm. The results were expressed as a percentage of the OD reading of the test sample (value), which was calculated as value = 100 × (S − NC)/(PC − NC) where S, NC, and PC are the OD of the test sample, the negative control, and the positive control, respectively. Serum samples were considered to be positive if they had a value of 40% or more, suspect if the value was between 30% and 40%, and negative if the value was <30%.

Exact confidence intervals (CI) for prevalence rates at the 95% level were calculated according to Sterne's method (Reiczigel, 2003). Prevalence rates were compared with the Fisher's exact test and differences were considered significant when $P < 0.05$.

3. Results and discussion

Except for *E. canis*, all evaluated tick-borne pathogens were found in non-pet dogs in the present study. Considering the haemoplasmas, *A. phagocytophilum* and *C. burnetii*, these were detected with high prevalence (Table 1). PCR and seropositivity did not correlate with the breed, sex or age groups of dogs (data not shown). Moreover, none of the evaluated infections was associated with geographical regions (i.e., positive samples were found from south-western to south-eastern Hungary). Co-infections with bacteria belonging to different genera were seldom detected (there were two dogs with concurrent haemoplasma and *A. phagocytophilum* PCR positivity and two with either simultaneous haemoplasma or *A. phagocytophilum* and *Coxiella* positivity), which is most likely due to the different routes (tick vectors) of infection and the epidemiology of the relevant agents. The prevalence rates of the evaluated agents were not significantly different between the three categories of non-pet dogs (Table 1).

3.1. Haemotropic Mycoplasma spp.

Altogether 14 dogs (11.1%, CI: 6.2–18%) had haemoplasma infection: all of them harboured '*Candidatus* M. haematoparvum', and 8 dogs (6.3%, CI: 2.8–12.1%) were co-infected with *M. haemocanis* (i.e., the latter species was not detected in single infection dogs). For *M. haemocanis* the copy numbers of DNA (reflecting bacterial loads) reached higher values (Table 1), which implies that in 7 out of 8 dual-PCR positive samples *M. haemocanis* predominated. The high prevalence of canine haemoplasmas is an unexpected finding because the geographical distribution

Table 1
Summary of molecular and serological investigation of tick-borne pathogens in three groups of non-pet dogs.

	Haemotropic Mycoplasma spp.		*Anaplasma phagocytophilum*	*Coxiella burnetii*		*Rickettsia* spp. (excluding *R. helvetica*)
	'*Candidatus* M. haematoparvum'	*M. haemocanis*				
	PCR positives/all tested (copy numbers)	PCR positives/all tested (copy numbers)	PCR positives/ all tested	PCR positives/ all tested	ELISA positives/ all tested	PCR positives/ all tested
Shepherd dogs	11/100 (1–3.8×10^3)	6/100 (2.1×10^1–1.3×10^4)	9/100	1/100	20/97	1/100
Hunting dogs	2/12 (4–1.2×10^2)	1/12 (2.1×10^4)	1/12	0/12	3/12	0/12
Stray dogs	1/14 (4)	1/14 (1.3×10^3)	4/14	0/14	2/14	0/14
In toto	14/126	8/126	14/126	1/126	25/123	1/126

of these pathogens corresponds to that of the vector, the brown dog tick *R. sanguineus* (Novacco et al., 2010), which is endemic to Mediterranean countries. Accordingly, canine haemoplasma species show decreasing prevalence towards the north in Italy (Novacco et al., 2010), and autochthonous infections are not reported north of the Mediterranean basin (Wengi et al., 2008). In contrast to this previous observation, dogs in the present study were sampled from a region with continental climate where *R. sanguineus* is not considered to be endemic (Hornok et al., 2013a). Supporting the absence of this tick species, all non-pet dogs evaluated here were negative for *E. canis*, another *R. sanguineus*-transmitted pathogen. Furthermore, housing in kennels (i.e., close contact between animals) which is an important predisposing factor of canine haemoplasma infection (Novacco et al., 2010), can also be excluded here as shepherd dogs of this study were kept extensively (outdoors). Therefore, other transmission route(s) and/or predisposing factors most likely have played a role in the present case. These may be associated with non-pet dogs and need to be studied further. As haemoplasma positivity was not related to any of the three groups of dogs evaluated in the present study, the mode of acquiring the infection may have been similar in shepherd, hunting and stray dogs.

3.2. *Anaplasma phagocytophilum*

Fourteen dogs (11.1%, CI: 6.2–18%) were PCR-positive for *A. phagocytophilum*. This prevalence rate for molecularly detectable bacteraemia was higher than in other European countries, such as Germany, Poland, Italy, Portugal and the UK (Kohn et al., 2011). The high prevalence of *A. phagocytophilum* in dogs in the present study may be explained by their constant exposure to vector ticks. In fact, shepherd dogs from the evaluated region are most frequently infested with the anthropophilic tick species *Ixodes ricinus* (Hornok et al., 2013b), which is the vector for *A. phagocytophilum*. Therefore, non-pet dogs should be considered as potential reservoirs and sentinels for this zoonotic pathogen.

3.3. *Coxiella burnetii*

Only one dog was found to be PCR-positive for *C. burnetii*, but 20.3% of serum samples (25 out of 123, CI: 13.6–28.5%) indicated seroconversion to the Q fever agent. Dogs were reported to play a role in the epidemiology of human Q fever (Buhariwalla et al., 1996), and contact with dogs represents a risk factor for acquiring the infection (de Rooij et al., 2012). This is the first report of Q fever seroprevalence in dogs from central-eastern Europe. In other (western and southern) European countries, the prevalence rates were usually lower (1–12%), with the exception of Switzerland (31%) (Boni et al., 1998). However, these differences may be partly explained by the lower sensitivities of formerly used serological assays (such as the complement fixation test). The high seroprevalence in the present study may be attributed to the feeding (keeping) mode of dogs in all three sample groups. These dogs regularly have access to products and body

fluids/parts of farm (game and wild) animals representing the most important source of *C. burnetii* infection (de Rooij et al., 2012), and Q fever is prevalent in the domestic ruminant stocks of Hungary (Gyuranecz et al., 2012).

3.4. Rickettsiae

One dog was found to be rickettsaemic and was infected with a species other than *R. helvetica* (the species could not be identified further as reflected by the relatively high Ct-value of 35.9). Independent of the rickettsia carrier status of ticks dogs are susceptible only to certain spotted fever group rickettsiae and infection with these is seldom detected due to transient (few days long) rickettsaemias (Norment and Burgdorfer, 1984). Among *Rickettsia* spp. in Europe, to the best of our knowledge, only *R. conorii* was reported to establish infection in dogs (Levin et al., 2012), and therefore it is the most likely candidate to be involved in the present case.

In summary, this is the first molecular evidence of autochthonous infection of dogs with haemoplasmas, *A. phagocytophilum*, *C. burnetii* and any rickettsiae in Hungary. Based on the present results non-pet dogs may have high prevalence of tick-borne infections (e.g., with haemoplasmas or *A. phagocytophilum*) and even bacteraemias seldom detected in pet dogs (as exemplified by *C. burnetii* and rickettsiae). These results suggest that non-pet dogs may serve as sentinels and potential reservoirs of tick-borne and zoonotic agents. The molecular and serological evaluation of non-pet dog samples may provide more appropriate information for infection risk assessment than similar data obtained from pet dogs.

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